Published in final edited form as:

Curr Fungal Infect Rep. 2016 ; 2016: 1–6. doi:10.1007/s12281-016-0256-3.

Evolution of Cryptococcal Antigen Testing: What is new?

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Abstract

Over the last decade, an upsurge in both the frequency and severity of fungal infections due to the HIV/AIDS epidemic and the use of immunosuppressive therapy has occurred. Even diagnostic methods like culture and microscopy, which have low sensitivity and longer turn-around-times are not widely available, leading to delays in timely antifungal therapy and detrimental patient outcomes. The evolution of cryptococcal antigen (CrAg) testing to develop inexpensive and more sensitive methods to detect cryptococcal antigen is significant. These newer tests employ immunoassays as part of point-of-care platforms, which do not require complex laboratory infrastructure and they have the potential to detect early disease and reduce time to diagnosis of cryptococcal infection. Advocacy for widely available and efficacious life-saving antifungal treatment should be the only remaining challenge.

Keywords

Cryptococcus; antigen; testing; lateral flow; assay; HIV; diagnosis; thermal contrast; Fungus; cryptococcal disease; cryptococcal meningitis

Introduction

Infection with the human immunodeficiency virus (HIV) and increased use of immunosuppressive therapy have led to an increase in both the frequency and severity of fungal infections [1]•. *Cryptococcus neoformans* and *Cryptococcus gatti* are responsible for

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Compliance with Ethics Guidelines

Conflict of Interest

Elizabeth Nalintya, Reuben Kiggundu, and David Meya declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

an estimated 700,000 cases of cryptococcal meningitis in sub Saharan Africa [2] and 7,800 cases of cryptococcal meningitis in North America in 2006 respectively [3]. Mortality from cryptococcal disease remains high even in the era of ART. Early diagnosis of cryptococcal infection is critical to improving clinical outcomes.

A notable increase in the prevalence of cryptococcal meningitis in the last decade has not been matched by improved diagnostics in resource limited settings where most of the infections occur. The low sensitivity of older test methods and delay in obtaining results has driven research for cheaper and more widely available sensitive diagnostics.

Traditional approaches to diagnosis include direct microscopic examination of clinical samples, histopathology, culture, and serology [1]•. However, new innovative technologies that use molecular and immunoassay point-of-care platforms have the potential to meet the needs of both resource-rich and resource-limited clinical environments [4]•. In this review, we describe the evolution of diagnostic techniques for cryptococcal infection focusing on CrAg testing and outline the current need and gaps in the area of cryptococcal infection testing.

Epidemiology of Cryptococcal meningitis

Cryptococcal meningitis (CM) is a severe life threatening illness caused by cryptococcus species, which mainly occurs in immune compromised individuals and rarely in immunocompetent persons and rarely in immunocompetent persons [5-7].

Cryptococcus is free living encapsulated saprophytic yeast. Human infections are caused by *Cryptococcus neoformans* and *cryptococcus gattii*. The epidemiology, clinical and molecular characteristics of these two species vary. *C. neoformans* is classified into *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D). Also identified are serotypes B and AD.[1]• *Cryptococcus neoformans* var. *grubii* causes most infections among HIV-infected persons [8, 9]. Ecologically, *C. neoformans* is found in soil contaminated with bird droppings, heart wood and homes of HIV-infected persons [10, 11]. *C. gattii* is classified into serotypes B and C. Previously known to be found predominantly in tropical and subtropical regions, outbreaks in Vancouver island, Canada and US Pacific North West have complicated our understanding of its ecological niche [12-14]. *C. gatti* infection predominantly occurs in immunocompetent patients [15].

The global burden of cryptococcal meningitis remains high despite advances in diagnosis and treatment. It is an AIDS defining illness, mainly occurring in patients with CD4 < 100 cells/μL [16, 17]. It is estimated that the mortality occasioned by CM among HIV infected patients is 50-70% [3]. The disease burden of CM parallels the HIV epidemic, with highest incidence and mortality in sub-Saharan Africa, South and Southeast Asia regions with high HIV prevalence and low access to ART [7, 18]. Annually, sub-Saharan Africa, and South and Southeast Asia account for 720,000 and 120,000 cases of CM respectively [3]. In addition to being the leading cause of meningitis in the sub-Saharan Africa region [7, 19-21], it accounts for 13-44% of all AIDS related deaths [22, 23], with mortality rates as high as 50-70% [3, 7, 19]. *C. neoformans* occurred in 5-15% of AIDS patients during the

peak of the HIV epidemic in Europe, United States and Australia, but the incidence of CM in these regions has declined partly due to access to HAART and antifungal use [15, 24-27].

Although HIV is the major risk factor for cryptococcal meningitis, immunosuppressive therapy, sarcoidosis and lymphoproliferative disorders are also associated with increased risk of developing cryptococcal meningitis [28].

Evolution of Cryptococcal Testing

The diagnosis of fungal infections in the past has relied primarily on techniques based on visualisation of the fungus, for example by direct microscopic examination of clinical samples, histopathology, and culture [4]•. These approaches require personnel with relatively high levels of specific mycology training [4]• and hence have a limitation for widespread use in resource limited settings. The increase in pathogenic fungi in the past decade has forced investigators to develop and apply new methods of fungal identification that go beyond classical phenotypic methods [4]•. As a consequence, there is increased emphasis on the use of molecular methods and antigen detection as surrogates for culture for the diagnosis of cryptococcal meningitis. Further still, old testing techniques lacked sensitivity and specificity and take too long to be clinically useful [1]•. We briefly describe the evolution of cryptococcal diagnostic techniques over time.

Direct microscopic examination of Clinical samples, Histopathology, and Culture

Culture has been the gold standard for diagnosis of cryptococcal disease and has characteristic advantages such as growing the specific organism and allowing for sensitivity testing in order to identify the most suitable therapy, however, the yield for most specimens is low and will usually be positive when the fungal burden is high. The turn-around-time using conventional culture media (Sabouraud Dextrose Agar and Mycosel agar; BD Diagnostic Systems) is usually more than 7 days, but could be positive in a few days among patients with high fungal burden and requires laboratory personnel with the requisite expertise [4]•. Fungal culture has evolved from conventional growth media to the use of birdseed (*Guizotia abyssinica*) agar for detection and rapid identification of *C. neoformans* [29]. This media has decreased the time to detection of most strains of *C. neoformans* from about several days to 72 hours - the time it takes for phenoloxidase activity to produce dark brown colored colonies. In a study comparing conventional media and birdseed agar by culture of 35 clinical samples from AIDS patients, the results showed 100% sensitivity and specificity with plates incubated at 30°C [4, 29]•. TOC (tween 80-oxgall-caffeic acid) agar has been used for identification of *C. neoformans* within 24 hours from previously isolated colonies [30]. However, it requires extended incubation of 3–5 days if used as the primary isolation medium. Detection of urease production for rapid recognition of *C. neoformans* [31] has also been attempted, but this method lacks specificity and needs to be followed by a more reliable method.

Microscopy is another fundamental technique whose sensitivity is dependent on the quality of the specimen and the experience of the laboratory personnel. Stains like India ink are used to stain specimens to ease visualisation. India ink staining, however, has limitations as described in a study comparing diagnostic techniques in Uganda. Sensitivity of India ink

microscopy was the lowest (86%) of any test and was highly dependent on fungal burden in CSF[4]. Sensitivity decreased to 42% (19/45) among persons with cerebrospinal fluid (CSF) cultures <1,000 colony forming units (CFU)/mL. Overall, 1 of 7.2 cryptococcal diagnoses was missed by India ink microscopy (negative predictive value of 80%; (95% CI 76%–84%). If India ink microscopy had been the only diagnostic test used, 8.8% of meningitis cases in Uganda would have been misdiagnosed. Among persons in Uganda who had India ink microscopy–negative results, *Cryptococcus* spp. remained the most common pathogen (20%). [32]••. India ink staining is also not suitable for diagnosis of invasive Cryptococcal disease, as this would require deep tissue biopsies.

Histopathology requires several stains enabling a more obvious appearance of *C. neoformans*. Classical stains used in histopathology include Gomori methenamine silver, periodic acid-Schiff, Gridley fungus, and hematoxylin and eosin stains [33]. Alternatively, Calcofluor white (CW) can be used with a fluorescent microscope to observe fungal elements in clinical samples. CW binds b -glycosidic linkages of polysaccharides in the fungal cell wall but also binds non-specifically to keratin and human connective tissue elements [34].

Antigen detection tests

These are tests that detect fungal antigen (*Cryptococcus neoformans and gatti*).

Latex agglutination—Diagnosis of CM was the first application of antigen detection for diagnosis of fungal infection that received widespread clinical use [35]. Antibodies were raised in rabbits against whole cryptococcal cells and passively coated onto latex beads. Termed latex agglutination, the assay detected glucuronoxylomannan (GXM), the major capsular polysaccharide of *C. neoformans*. GXM is shed in large amounts into blood and CSF during the course of cryptococcal meningitis. GXM occurs in four major serotypes: A, B, C, and D and a hybrid serotype AD [1]•. Studies done in 2012 to validate this test against newer test are summarised in Table 1 [32]••.

Most manufacturers (e.g., IMMY, Meridian Biosciences Inc., and Bio-Rad) propose and recommend use of pronase to reduce false-positive results caused by the presence of rheumatoid factors in the specimen especially in serum [4]•.

Lateral flow assay

A ground breaking landmark for cryptococcal antigen testing was the development of a lateral flow immunoassay (dipstick) (CrAg LFA). It was developed using a cocktail of monoclonal antibodies that were formulated to be reactive with all GXM serotypes [1, 37]•. This dipstick test uses gold-conjugated, monoclonal antibodies impregnated onto an immunochromatographic test strip to detect cryptococcal capsular polysaccharide glucuronoxylomannan antigen for all 4 *C. neoformans* serotypes (A–D)[37]. If cryptococcal antigen is present in a specimen, suspended, gold-conjugated antibodies bind to the antigen. The gold-antibody- CrAg complex migrates by capillary action up the test strip, interacts with immobilized monoclonal antibodies against the antigen and forms a band. The LFA kit contains immunochromatographic test strips, positive controls, and assay diluent that can be

stored at room temperature for ≤2 years. To perform the LFA, 1 drop of diluent (≈40 μL) is added to a container with 40 μL of patient specimen. The dipstick is inserted into the container and incubated at room temperature for 10 min [32]••.

In a review article evaluating the LFA, seven conference abstracts and two full-length published articles through August 2012 were reviewed. Six abstracts and the two full-length articles reported data on serum specimens and five abstracts included data on CSF specimens. The median sensitivity using serum was 100% (95.6%, 100%) and the median specificity was 99.5% (95.7%, 100%). Using CSF specimens, the median sensitivity was 100% (96.2%, 100%) and the median specificity was 97.7% (70.4%, 100%) [4]•. In another large scale evaluation of the lateral flow assay method, 1,000 specimens (589 serum and 411 CSF specimens) were tested in parallel at the ARUP laboratories a national reference laboratory under the pathology department of university of Utah. Comparison of Meridian EIA vs IMMY LFA showed 97.8% agreement (positive agreement 71.8, negative agreement 97.7%), kappa 0.82(0.75-0.9). In conclusion, the IMMY assays showed excellent overall concordance with the Meridian EIA. Assay performance differences appear to be related to issues of analytic sensitivity and serotype bias [38]••. Serotype sensitivity of the LFA has previously been assessed and the CrAg LFA found to have high sensitivity for GXM of all four serotypes, with A = B > C > D. The observed sensitivity of the CrAg LFA was greater than was previously reported for currently available CrAg immunoassays in latex agglutination or enzyme immunoassay formats [37].

Another study comparing four assays assessed detection of cryptococcal antigen in serum (n=634) and CSF (n=51). When compared to latex agglutination, the sensitivity and specificity of the Premier EIA, Alpha CrAg EIA and CrAg LFA were 55.6/100, 100/99.7 and 100/99.8%, respectively, from serum samples. There was 100% agreement among the four tests for CSF, with 18 samples testing positive by each of the assays [39]•.

The LFA is a semi quantitative test that can be used to measure disease burden by determining the CrAg titers for positive results. These have been found to be informative for patients with asymptomatic antigenemia and for patients with high titers the risk of cryptococcal meningitis and death is higher than those with lower titers as observed in a recently concluded CrAg screening study in Uganda. Contrary to our earlier understanding regarding the lack of a role for CrAg titers in treatment and risk stratification for symptomatic CM, among asymptomatic CrAg positive patients titers might have a role in improving patient clinical management and hence patient outcome. Further assessment of this can be done with thermal contrast measurement as described below.

Thermal Contrast Measurement of CrAg Titer

This laser thermal contrast method was suggested in 2012 and in a study done in Uganda it was used to provide quantification of the LFA in comparison with semi quantitative CrAg LFA titers by using the heat signature of laser-irradiated gold used in the LFA. To detect gold nanoparticles conjugated to monoclonal antibodies on the LFA line, the line was irradiated with a 0.01 W laser (532 nm, diode pumped; Millenia, Santa Clara, CA, USA) for 30 seconds, and temperature change (thermal contrast) was recorded with an infrared camera (A20; FLIR ThermoVision, Portland, OR, USA), as described [40]•. Three spots on each

horizontal LFA line were irradiated and the average maximum temperature change was calculated. An antigen titer was calculated from the thermal contrast by using a calibration curve established by 2-fold serial dilutions of 3 specimens in triplicate with known CrAg LFA titers ($R^2 = 0.97$). This study demonstrated that a novel technique, laser thermal contrast, had 92% accuracy in quantifying CrAg titers from 1 LFA strip to within <1.5 dilutions of the actual CrAg titer by serial dilutions ($R = 0.91$, $p<0.001$). LFA performance was more sensitive than that of any other diagnostic test. Conversely, the worst performing test was India ink microscopy, which is the most common cryptococcal diagnostic test in Africa, despite missing 1 in 7 cryptococcal diagnoses and having only an 80% negative predictive value in our cohorts [32]••.

Implications for the Future

Testing for cryptococcal disease has evolved over the last few years with particular improvements in CrAg testing. The development of the lateral flow assay could revolutionize diagnosis and management of cryptococcal disease. The test offers the ability to perform cryptococcal antigen testing at point-of-care, without the additional requirement of complex laboratory infrastructure especially in sub-Saharan Africa, where cryptococcal disease is prevalent.

The persistence of cryptococcal antigen remains an issue especially among patients who present with recurrence of symptoms and signs of meningitis (having had a prior episode of cryptococcal meningitis) and are antiretroviral therapy-experienced. In these cases, the clinician should be guided by use of CSF culture to differentiate between relapse (or new infection) and paradoxical cryptococcal immune reconstitution inflammatory syndrome (IRIS), with sterile cultures during IRIS, despite a positive CrAg test.

The experimental methods for cryptococcal antigen testing including thermal contrast could be developed further into cheaper bedside tests that could increase the repertoire of cryptococcal diagnostics in the future.

Finally, the diagnosis of early disease has become an important aspect of cryptococcal antigen testing with high sensitivity of the lateral flow assay providing the capability to detect lower quantities of antigen. An ongoing study on cryptococcal antigen testing among asymptomatic patients with CD4 counts <100 cells/μL in Uganda suggest that patients with higher CrAg titers >1:160 have a higher risk of death [41]. This is important as it presents an opportunity to study tailored therapy if one can determine the antigen titer at the time of CrAg testing. Studies conducted in South Africa have shown that CrAg screening of individuals initiating ART and preemptive fluconazole treatment of CrAg-positive patients resulted in markedly fewer cases of CM compared with historic unscreened cohorts. It has also been found to be a cost effective intervention and several modifications of dose and duration of the recommended Fluconazole therapy has led to improved survival [42-47].

For industry, the future should focus on developing a modified lateral flow assay that can further provide a semi quantitative CrAg titer which could be a separate band, for example >1: 160 that could be read off the test strip at the same time the LFA is being read for qualitative positive results. This would enable clinicians to study different treatment

regimens especially for asymptomatic patients who could benefit from pre-emptive antifungal therapy.

Conclusion

In keeping with new developments in the diagnosis of infectious diseases, the development of the lateral flow assay as a point-of-care test for detecting cryptococcal antigen is a huge leap forward and introduces a new paradigm in the management of cryptococcal disease. The ability to screen for cryptococcal antigen, especially prior to initiating antiretroviral therapy among HIV-infected patients and pre-emptively treating those with ‘early’ cryptococcal disease using antifungal therapy to prevent overt and symptomatic cryptococcal disease will save health care costs especially for resource poor countries by eliminating the need for complex laboratory infrastructure. The focus should now be on making this test more widely available, implementing national CrAg screening programs and advocating for more efficacious antifungal treatment regimens that would minimize mortality and morbidity occasioned by HIV infection and cryptococcal co-infections.

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Table 1

Performance characteristics of cryptococcal diagnostic assays in persons with suspected meningitis, Uganda and South Africa*

		Number positive/Number tested (%)			
Diagnostic test	**N**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
CSF culture	806	459/510 (90.0)	296/296 (100.0)	459/459 (100.0)	296/347 (85.3)
100-µL volume	524	309/328 (94.2)	196/196 (100.0)	309/309 (100.0)	196/215 (91.2)
10-µL volume	282	150/182 (82.4)	100/100 (100.0)	150/150 (100.0)	100/132 (75.8)
India ink microscopy	805	438/509 (86.1)	288/296 (97.3)	438/446 (98.2)	288/359 (80.2)
CrAg LFA	666	435/438 (99.3)	226/228 (99.1)	435/437 (99.5)	226/229 (98.7)
CrAg latex (Meridian)	279	176/180 (97.8)	85/99 (85.9)	176/190 (92.6)	85/89 (95.5)
CrAg latex (Immy)	749	452/466 (97.0)	283/283 (100.0)	452/452 (100.0)	283/297 (95.3)

PPV- Positive predictive value; NPV- Negative predictive value; CrAg- Cryptococcal antigen; LFA- lateral flow assay; CSF- cerebrospinal fluid